

## Identification of 5' Regulatory Elements of the PDGF-A Chain Gene, and Interaction with Single-stranded DNA Binding Protein

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### ABSTRACT

The expression of platelet-derived growth factor (PDGF) is controlled in a very complicated manner. To clarify the mechanism of regulation of the PDGF-A chain gene, deletion analysis of the 5'-flanking region was performed. We identified a positive regulatory element 25 base pairs (bp) upstream of TATAA, a negative element 135 bp upstream, a positive element 223 bp upstream and a negative element further upstream. These regulatory sites of the PDGF-A chain gene may be involved in tissue specificity, developmental regulation, and transformation. In addition, our analysis suggested the presence of a strand non-specific single-stranded DNA binding nuclear protein in the positive regulatory element 25 bp upstream of TATAA.

**Key words:** PDGF-A, 5' regulatory elements, single-stranded DNA binding protein

Human platelet-derived growth factor (PDGF) is present in  $\alpha$  granules of platelets and is a heterodimeric protein consisting of A and B chains. Since PDGF proliferates mesodermal cells and induces migration of leukocytes, smooth muscle cells, and fibroblasts, its association with wound healing and atherosclerosis has attracted attention<sup>4)</sup>. The PDGF-A chain is widely expressed in tumor cells<sup>1)</sup>, suggesting the oncogenicity of this chain. Genomic clones of the PDGF-A chain have been isolated and sequenced<sup>27)</sup>, and the promoter region has been determined<sup>33)</sup>. However, the mechanism of its gene regulating action has not yet been clarified. Understanding the mechanism of genetic regulation may provide clues toward the clarification of the various pathologic conditions mentioned above. In this study, we identified positive and negative regulatory elements in the 5' flanking region by chloramphenicol acetyl transferase (CAT) assay<sup>7)</sup> and demonstrated the presence of a nuclear protein that predominantly binds to single-stranded DNA in the positive element immediately upstream of the promoter.

### MATERIALS AND METHODS

**Plasmids construction:** As shown in Fig. 1, various deletion mutants were constructed on the 5' side using various restriction enzymes. CAT, Xb-S, SstI-1~6 were constructed by partial di-

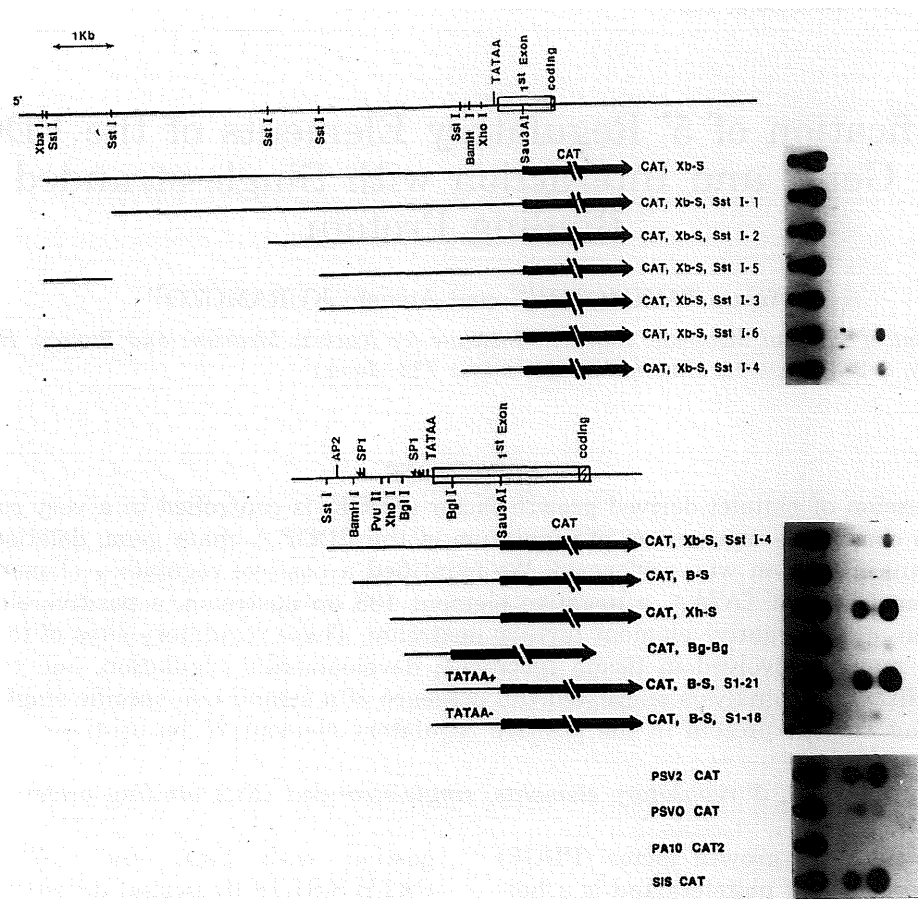
gestion with SstI, and CAT,B-S,S1-21 and CAT,B-S,S1-18 by partial digestion with nuclease S1, and the sites of splitting were confirmed by sequencing. CAT,Sy17 and CAT,Sy108 were constructed by artificial synthesis of the sites shown in Fig. 2. CAT,Sy17, S1-18 and CAT,Sy108,S1-18 were produced by connecting the fragments of CAT,Sy17 and CAT,Sy108, respectively, to a site upstream of CAT,B-S,S1-18 using a linker. Whether each fragment was correctly constructed was confirmed by sequencing. Each constructed fragment was inserted into the site upstream of the CAT gene in the 5'-3' orientation.

**DNA transfection and CAT assay:** About 24 hours before transfection, RD cells (human embryonal rhabdomyosarcoma cell line) were seeded at  $5.5 \times 10^5$  per 100-mm Petri dish. 20  $\mu$ g of each plasmid was transfected by the calcium phosphate method<sup>8)</sup>. After 3 hr, the cells were treated with 15%(vol/vol) glycerol in 20mM Hepes buffer for 3min, washed, incubated for 48hr, and harvested, and 100  $\mu$ g of lysates was assayed for CAT activity<sup>7)</sup>.

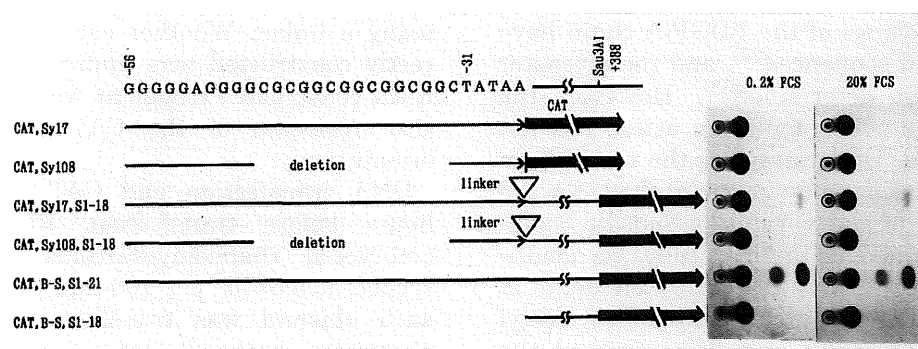
**Gel Retardation Assay:** Nuclear protein was extracted by the method of Shapiro et al<sup>31)</sup>. After hypotonic treatment the suspended cells were destroyed using a Dounce homogenizer. The nucleus was obtained by centrifugation, and the

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**Fig. 1.** Deletion analysis of 5'-flanking region RD cells were transfected with plasmids constructed with various deletion mutants on the 5' side (left figure). After 48 hr of incubation, the cells were harvested, lysed and assayed for CAT activity (right figure). Details of the experiments are shown in Materials and Methods.



**Fig. 2.** Deletion analysis of 5'-flanking region RD cells were transfected with plasmids by the calcium phosphate method, and CAT activity was determined after 48 hr. -33 ~ -46 upstream of TATAA were deleted in CAT.Sv108.S1-18.

nuclear protein was extracted with ammonium sulfate. For the gel retardation assay,  $^{32}\text{P}$ -DNA fragment (25 k cpm) labeled by T4 polynucleotide kinase, 5 $\mu\text{g}$  of nuclear protein, and 5 $\mu\text{g}$  of poly (dI-dC) were mixed in 25 $\mu\text{l}$  of binding buffer (10mM Hepes, pH 7.9; 60mM KCl; 7.5mM  $\text{MgCl}_2$ ; 0.1mM EDTA; 1mM DTT; and 10% glycerol), incubated at room temperature for 30 min, and subjected to electrophoresis (10V/cm) with 5%

polyacryl amide gel.

## RESULTS

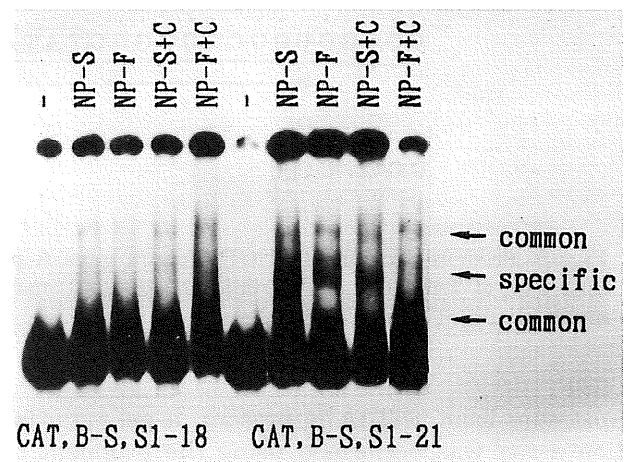
Deletion analysis of the 5'-flanking region: As shown in Fig. 1, CAT,B-S,S1-18 without the TATAA sequence had no CAT activity, suggesting that TATAA in this fragment also acts as a promoter in CAT plasmids. Interestingly, CAT,B-S,S1-21 including only 25 base pairs(bp)

upstream from TATAA showed high CAT activity. However, CAT activity almost disappeared in CAT,Bg-Bg (135 bp upstream from TATAA). Unlike the other fragments, the 3' end of CAT,Bg-Bg terminates in a BglI site and, therefore, the effects of the BglI-Sau 3AI fragment in the 5'-untranslated region (UTR) can not be ignored. However, our analysis of 5'-UTR showed positive CAT activity in 5'-UTR up to BglI as in 5'-UTR up to Sau3AI<sup>34</sup>. The negative effect on CAT activity observed in CAT,Bg-Bg seemed to be due to the 5' side fragment. High CAT activity re-appeared in CAT,Xh-S (223 bp upstream from TATAA), but CAT activity was re-inhibited in further upstream fragments. Thus, we identified a positive fragment 25 bp upstream from TATAA, a negative fragment 135 bp upstream, a positive fragment 223 bp upstream, and a negative fragment further upstream.

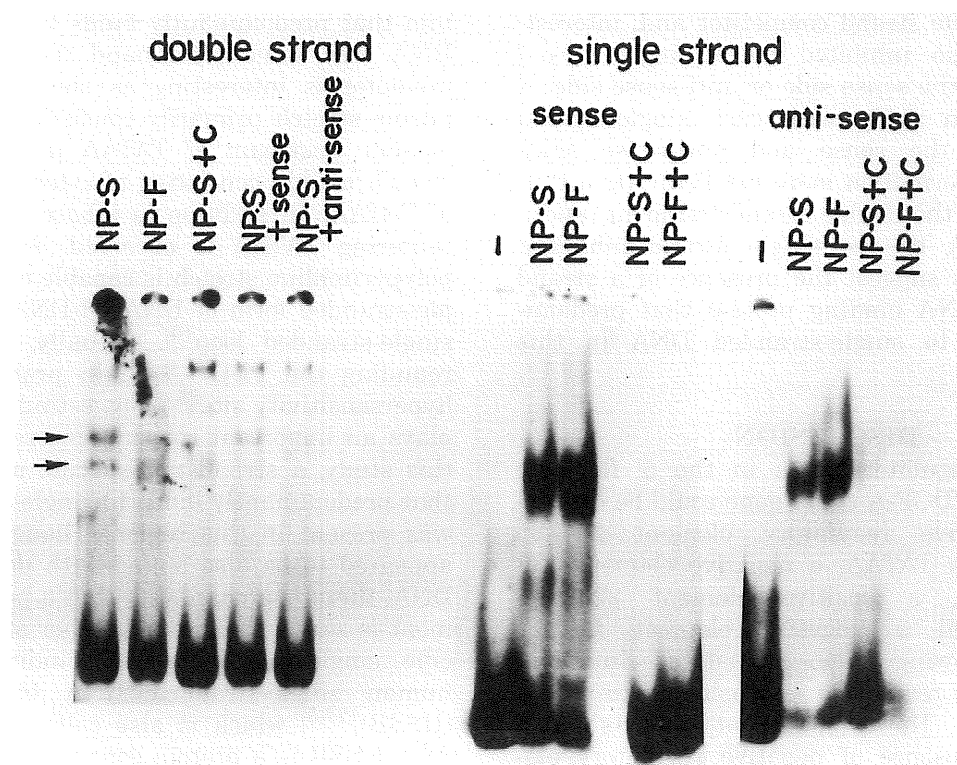
Since high CAT activity was observed in the fragment 25 bp upstream from TATAA, deletion mutants were produced as shown in Fig. 2. CAT activity in CAT,Sy17,S1-18 was lower than that in CAT,B-S,S1-21, since a linker was inserted immediately downstream of TATAA but was definitely present as compared with CAT,B-S,S1-18. However, CAT,Sy108,S1-18, in which -33 ~ -46 upstream of TATAA were deleted, showed no CAT

activity. This suggests the important role of this short TATAA-upstream fragment in transcription.

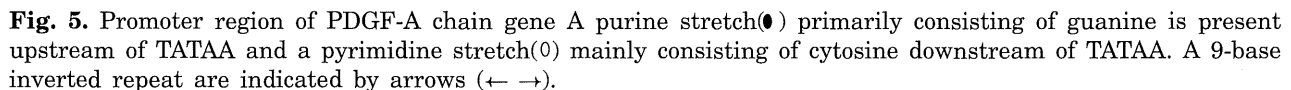
Gel retardation assay: To clarify whether a nuclear protein binding to the fragment 25 bp



**Fig. 3.** Gel retardation assay Gel retardation assays were performed using nuclear proteins extracted from serum starved RD cells (NP-S) and from RD cells treated with 20% FCS and cycloheximide (NP-F).C: competitor(15 times more than the probe)



**Fig. 4.** Gel retardation assay Gel retardation assays were performed using nuclear proteins extracted from serum starved RD cells (NP-S) and from RD cells treated with 20% FCS and cycloheximide (NP-F).A gel retardation assay with double stranded fragment 25 bp upstream from TATAA showed a retarded band(→). This band was inhibited by a double strand competitor(+C), and a single strand competitor on the sense side (+sense) or anti-sense side(+anti-sense).



## DISCUSSION

In the positive regulatory element immediately upstream of the promoter in the PDGF-A chain, we demonstrated the presence of a nuclear protein that predominantly binds to single-stranded DNA. The structure around the PDGF-A chain promoter is interesting as shown in Fig. 5. A purine stretch primarily consisting of guanine is present upstream of TATAA and a pyrimidine stretch mainly consisting of cytosine downstream of TATAA. In addition, a 9-base inverted repeat centering TATAA is observed. A polypurine or polypyrimidine stretch is capable of forming a triple-stranded form of DNA (H-DNA) containing a single-stranded loop<sup>10)</sup>. Actually, the area surrounding the PDGF-A chain promoter is a S1 hypersensitivity site<sup>35)</sup>, suggesting that this area plays an important role in gene regulation<sup>17)</sup>. In this study, a strand non-specific nuclear protein that predominantly binds to single-stranded DNA was present in this region. Though this protein appeared to be also bound with double-stranded DNA, there is a possibility that a part of the fragment is single stranded. Another strand non-specific single-stranded DNA binding protein is human single-stranded DNA binding protein (HSSB)<sup>12,36)</sup> which is also called RF-A<sup>5)</sup> or RP-A<sup>37)</sup>. HSSB is a protein consisting of 70, 32, and 14 KDa subunits. The 70 KDa subunit binds to single-stranded DNA. HSSB not only stabilizes the bound single-stranded DNA but also activates DNA polymerase<sup>13,14)</sup> and helicase<sup>30)</sup>, suggesting its involvement in replication and repair. However, there are no reports that suggest the

involvement of HSSB in transcription. Whether HSSB is identical to the single-stranded DNA binding protein observed in the PDGF-A chain in this study is also an interesting problem. Recently, single-stranded DNA binding proteins involved in transcription have been reported, but they are sequence (strand) specific<sup>16,21,22,24,28</sup>. The strand non-specific DNA binding protein that binds to single-stranded DNA 5' upstream in the PDGF-A chain observed in this study may play the following roles. (1) Maintenance of the promoter region in the single-stranded state, (2) facilitation of binding of other single-stranded DNA binding proteins, and (3) activation of proteins involved in transcription, facilitating transcription. A recent study has shown that TFIID, which binds to TATAA and initiates transcription, also has single-stranded DNA binding activity<sup>9</sup>. The 25-base fragment upstream of TATAA may form a single-stranded loop, binds to the strand non-specific single-stranded DNA binding protein to continuously send positive signals regardless of the effects of the serum.

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